ZYMO RESEARCH

THE Epigenetics COMPANY™

5-mC DNA ELISA Kit

Accurate quantitation of 5-mC in any DNA sample in less than 3 hours.

Highlights

- High-throughput detection of global 5-methylcytosine (5-mC) in DNA.
- Simple streamlined protocol validated for several species including human, mouse, and more.

Catalog Numbers:
D5325 & D5326

Scan with your smart-phone camera to view the online protocol/video.

tech@zymoresearch.com www.zymoresearch.com Toll Free: (888) 882-9682

Table of Contents

Product Contents

5-mC DNA ELISA Kit	D5325 (1 x 96 wells)	D5326 2 x 96 wells)	Storage Temperature
5-mC Coating Buffer	15 ml	30 ml	4 °C
5-mC ELISA Buffer	250 ml	250 ml x 2	4 °C
Anti-5-Methylcytosine (0.5 µg/µl)	15 µl	30 µl	-20 °C
Secondary Antibody (1 µg/µl)	15 µl	30 µl	-20 °C
HRP Developer	15 ml	30 ml	4 °C
Negative Control (100 ng/µl)	50 µl	50 µl	-20 °C
Positive Control (100 ng/µl)	50 µl	50 µl	-20 °C
96-well plate	1 plate	2 plates	Room temp.

Note - Integrity of kit components is guaranteed for up to up to six (6) months from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide the highest performance and reliability.

Specifications

- **Sample Source –** Purified genomic DNA, plasmid DNA, PCR amplification products, or DNA fragments in water, Tris-EDTA, or similar.
- **DNA Quantity –** The protocol is optimized for 100 ng input DNA/well. Compatible with DNA in the range of 10-200 ng.
- **Detection –** ≥ 0.5% 5-methylcytosine (5-mC) per 100 ng single-stranded DNA.
- **Equipment Required –** Incubator and ELISA plate reader. A multi-channel pipettor is recommended. An automated plate washer may be used for blocking and washing steps.

Product Description

The ability to efficiently detect and quantify DNA methylation (i.e., 5-methylcytosine) has become essential for epigenetic-based research. To date, several methods have been developed for this purpose including high-performance capillary electrophoresis, bisulfite sequencing, and methylated DNA immunoprecipitation.

The **5-mC DNA ELISA Kit** is a convenient and powerful tool that allows the researcher to accurately quantitate 5-mC in any DNA sample in less than 3 hours. The kit features a unique **Anti-5-Methylcytosine** monoclonal antibody that is both sensitive and specific for 5-mC. The assay is compatible with a wide range of input DNA from vertebrate, plant, and microbial sources as well as PCR amplicons and fragmented DNA. Percent 5-mC in a DNA sample can be accurately quantified from a standard curve generated with specially designed controls included with the kit. This fast, streamlined workflow is also ideal for high-throughput analyses.

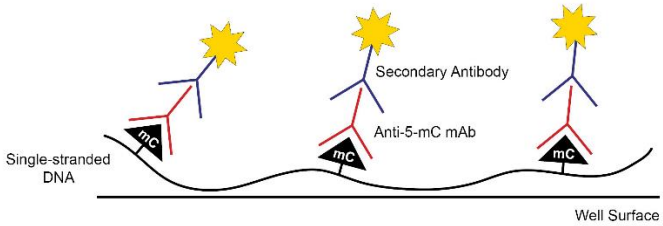

Figure 1. The 5-mC DNA ELISA Kit utilizes the indirect ELISA technique in its workflow. Denatured**,** single-stranded DNA samples are coated on the well surfaces in 5-mC Coating Buffer. Anti-5-Methylcytosine monoclonal antibody (Anti-5-mC mAb) and the HRP-conjugated Secondary Antibody are prepared in 5-mC ELISA Buffer and added to the wells. Detection of 5-mC occurs after addition of the HRP Developer.

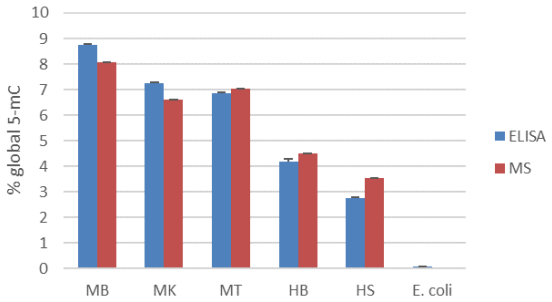

Figure 2. The 5-mC DNA ELISA Kit can quantify 5-mC in numerous DNA samples with close correlation to LC-MS. 100 ng of genomic DNA from mouse brain (MB), mouse kidney (MK), mouse thymus (MT), human brain (HB), human spleen (HS), and E. coli ER2925 were used to coat wells, in triplicate. Percent 5-mC was calculated using the logarithmic equation of the line from the standard curve that was constructed with the Negative Control and the Positive Control (see Appendix, page 5). The percent 5-mC calculated in DNA samples using the 5-mC DNA ELISA Kit (ELISA) strongly correlates to mass spectrometry (MS) data of 5-mC found in the respective gDNA sample.

Experimental Conditions

- ✓ All DNA must be denatured (single-stranded) for use with the kit (refer to DNA Coating steps on page 5). The protocol is optimized for the detection of 5-mC in 100 ng of double stranded DNA per well that has been denatured. All samples should be assayed in duplicate (meaning a total of 200 ng of DNA/sample will be used with this assay). However, depending on your experimental design, 10 to 200 ng of sample DNA can be used in the assay.

  Note: When using inputs other than 100ng per well, the amount of control DNA used must be adjusted to equal the amount of sample used. This will ensure accurate % 5-mC quantification.

- ✓ The **Negative** and **Positive Controls** consist of double stranded DNA at a concentration of 100 ng/µl and can be used for the detection/quantification of 5-mC in DNA. For 5-mC detection, both controls should be assayed. For 5-mC quantification, the **Negative Control** should be mixed with the **Positive Control** at different ratios to construct a standard curve (See Appendix, page 7). All standards should be assayed in duplicate.

- ✓ **Secondary Antibody** is a horseradish peroxidase (HRP) conjugate and supplied at a concentration of 1 ug/µl. Avoid freeze/thaw cycles; if necessary make aliquots of the antibody and keep at -20°C for long term. Store thawed antibody at 4°C for short periods of time (~1 week).

- ✓ **Anti-5-Methylcytosine** is supplied at a concentration of 0.5 ug/µl. Avoid freeze/thaw cycles; if necessary make aliquots of the antibody and keep at -20°C for long term. Store thawed antibody at 4°C for short periods of time (~1 week).

Protocol

This protocol is optimized for 100 ng of DNA per well.

Duplicate samples are recommended for accurate 5-mC detection and quantification.

5-mC Control Preparation:

For 5-mC Detection:

The presence or absence of 5-mC can be determined by comparing the absorbance of samples to **Negative** (0% methylation) and **Positive** (100% methylation) **Controls** assayed in parallel with experimental samples.

For 5-mC Quantification:

To quantify the percentage of 5-mC in a DNA sample, a standard curve[1] must be generated. This is done by preparing mixtures[2] of the **Negative Control (100ng/µl)** and **Positive Control (100 ng/µl)** to generate standards of known 5-mC percentage (see table below). These standards should be assayed in parallel on the same plate with the samples.

% 5-mC	Negative Control (100 ng/µL)	Positive Control (100 ng/µL)
0%	10.0 µL	0 µL
5%	9.5 µL	0.5 µL
10%	9.0 µL	1.0 µL
25%	7.5 µL	2.5 µL
50%	5.0 µL	5.0 µL
75%	2.5 µL	7.5 µL
100%	0 µL	10 µL

This table highlights the preparation of seven mixtures using the **Negative Control** and **Positive Control** to be used to generate a standard curve. Total volume for each is 10 µl at a concentration of 100 ng/µl.

[1] A new standard curve should be generated for each assay.
[2] The number of standard curve mixtures for 5-mC quantification can vary. In the example given in the table, seven mixtures were prepared. Leftover mixtures can be frozen at or below -20ºC for future use.

DNA Coating:

1. Remove the necessary number of well strips[3] to assay DNA samples and controls.
2. Add 100 ng of each DNA[4] to a PCR tube and bring the final volume to 100 µl with **5- mC Coating Buffer**.
3. Denature the DNA at 98°C for 5 minutes in a thermal cycler. After denaturation, transfer immediately to ice for 10 minutes.
4. Add the entire volume (100 µl) denatured DNAs to the wells of the plate, cover with foil, and incubate at 37°C for 1 hour.

Blocking:

1. Discard the buffer from the wells[5].
2. Wash each well 3 times with 200 µl of **5-mC ELISA Buffer.** *Discard the buffer after each wash.*
3. Add 200 µl of **5-mC ELISA Buffer** to each well. Cover the plate with foil and incubate at 37°C for 30 minutes.

Antibody Addition:

1. Discard the buffer from the wells.
2. Prepare an antibody mix[6] consisting of **Anti-5-Methylcytosine** and **Secondary Antibody** in **5-mC ELISA Buffer** according to the following table.

Reagent	Dilution	Volume (µL)	Example (18 wells)
5-mC ELISA Buffer	**N/A**	**(# wells+ 2) × 100**	**2,000 µL**
Anti-5-Methylcytosine	**1:2,000**	**Buffer volume ÷ 2,000**	**1 µL**
Secondary Antibody	**1:1,000**	**Buffer volume ÷ 1,000**	**2 µL**

3. Add 100 µl of this antibody mix to each well. Cover the plate with foil and incubate at 37°C for 1 hour.

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[3] The well strips should be stored in a clean, dry, dark place for later use.
[4] Make sure that the volume of the DNA added to the 5-mC Coating Buffer does not exceed 20% of the final volume.
[5] Tap out any remaining buffer onto a paper towel after emptying a well.
[6] The antibody mix can be prepared during the blocking step and kept on ice until it is needed.

Color Development:

1. Discard the antibody mix from the wells.
2. Wash each well 3 times with 200 µl of **5-mC ELISA Buffer.**
3. Add 100 µl of **HRP Developer** to each well. Allow color to develop for 10-60 minutes[7] at room temperature.
4. Measure absorbance at 405 nm[8] using an ELISA plate reader.

[7] The development time will depend on the temperature of the HRP Developer. Development time may vary according to experimental design as well.
[8] Alternatively, absorbance readings between 405-450 nm can be measured.

Appendices

5-mC Quantification using Standard Curve

If standards were assayed in parallel with the experimental samples, a logarithmic standard curve can be generated to calculate the percentage of 5-mC in the sample. The absorbance for each mixture must be plotted as a function of Absorbance @ 405 nm (Y-axis) vs. % 5-mC (X-axis). Using a logarithmic curve, the equation y = ln(x) + b will be generated. The 5-mC percentage of the samples can then be determined (see equation below) based on the absorbance measured.

$$\% \text{ 5-mC} = e^{\left\{\frac{(\text{Absorbance - y-intercept})}{\text{Slope}}\right\}}$$

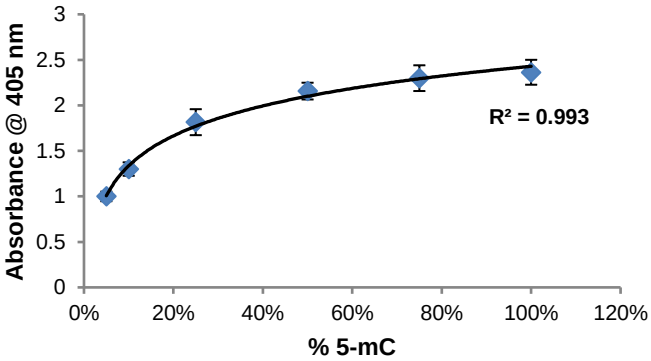

Figure 3. Standard curve generated with DNA mixtures. The curve was generated using the absorbance values of the mixtures indicated in the table above. A logarithmic relationship was observed with a correlation of 0.99.

Calculating CpG and Global Methylation

The %5-mC calculated from the standard curve above results in the relative %5-mC between samples. Since the Positive and Negative Control DNAs are derived from *Escherichia coli* gDNA, the density of CpG dinucleotides may vary from the organism of interest (OOI). Thus, to report CpG methylation % (%5mC/CpG) or global methylation % (%5-mC/total cytosine), the difference in CpG density and cytosine density of the OOI to *E. coli* genome must be considered. Table 1 lists common species and their genomic characteristics that can be referenced to calculate CpG methylation and global methylation.

1. Calculate the relative %5-mC for each sample using the standard curve equation as described above.

2. To determine %CpG methylation (%5mC/total CpG sites), multiply the relative %5-mC by the fold difference in CpG density of the *E. coli* and OOI:

%CpG Methylation = Relative %5mC × Fold Difference

3. To determine global cytosine methylation (%5mC/total cytosine), multiply the calculated %CpG methylation by the OOI's cytosine density:

Global %5mC = % CpG Methylation × Cytosine Density

Table 1. Cytosine and CpG information for common species. This table can be referenced for calculating %CpG and global methylation for the organism of interest.

Organism of Interest (OOI)	Genome Length (bp)	Total Cytosine	# of CpG Sites	CpG Density (# of CpG / Length)	Fold Difference (E. coli vs OOI)	Cytosine Density (Cytosine / Length)
Arabidopsis	237,920,282	42,859,753	5,567,714	0.0234	3.193	0.180
E. coli	4,639,675	1,179,554	346,670	0.0747	1.000	0.254
Human hg19	3,137,161,264	585,012,752	28,700,086	0.0091	8.167	0.186
Mouse mm9	2,654,895,218	534,142,064	21,342,492	0.0080	9.295	0.201
Pig	2,808,525,991	525,183,245	30,460,512	0.0108	6.889	0.187
Rabbit	2,737,490,501	569,377,656	36,000,387	0.0132	5.682	0.208
Rat rn4	2,834,127,293	572,296,669	24,799,282	0.0088	8.539	0.202
Zebrafish danRer7	1,412,464,843	258,711,448	25,251,167	0.0179	4.180	0.183

Example:

The relative %5mC for a mouse sample was calculated to be 2.50%.

The fold difference in CpG density of *E. coli* to mouse is 9.295. Therefore, the CpG methylation % was calculated to be 23.2%.

%CpG Methylation = Relative %5mC × Fold Difference = 2.50% × 9.295 = **23.2%**

The global methylation % for the sample was then determined using the density of the mouse genome (0.201). This mouse sample had 4.67% global methylation of cytosines.

Global %5mC = % CpG Methylation × Cytosine Density = 23.2% × 0.201 = **4.67%**

Ordering Information

Product Description	Catalog No.	Size
5-mC DNA ELISA Kit	D5325 D5326	1x96 wells 2x96 wells

Individual Kit Components	Catalog No.	Amount
5-mC Coating Buffer	D5325-1-15 D5325-1-30	15 ml 30 ml
5-mC ELISA Buffer	D5325-2-250	250 ml
Anti-5-Methylcytosine (0.5 ug/µL)	A3002-15 A3002-30	15 µl 30 µl
Secondary Antibody (1 ug/µL)	D5325-3-15 D5325-3-30	15 µl 30 µl
HRP Developer	D5425-4-15 D5425-4-30	15 ml 30 ml
Negative Control (100ng/µL)	D5325-5-1	50 µl
Positive Control (100 ng/µL)	D5325-5-2	50 µl
96-well ELISA plate (12x8 Strips)	C2020	1 plate

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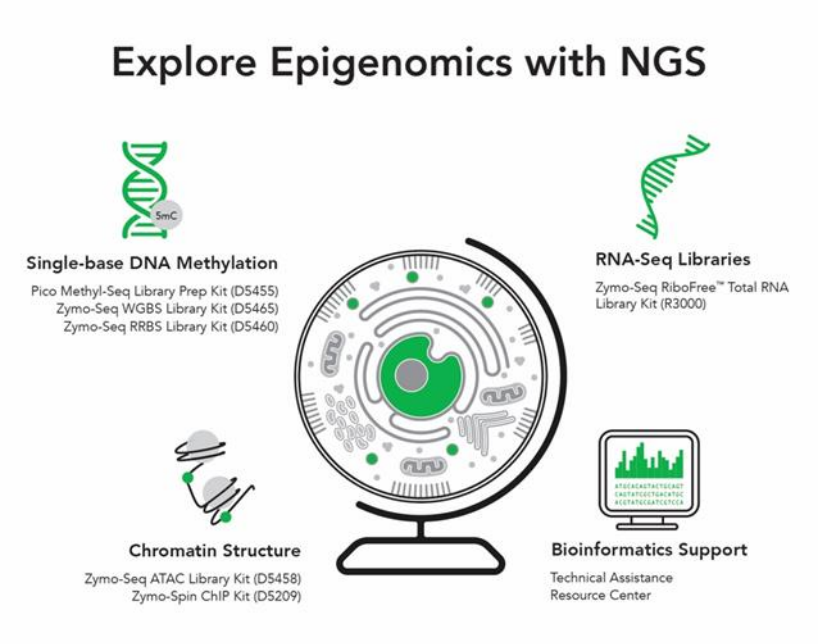

Notes

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